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I, JONNE YABSLEY, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. PS 2834 for a patent by MONASH UNIVERSITY as filed on 07 June 2002.

WITNESS my hand this
Seventeenth day of June 2003

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Regulation 3.2

A U S T R A L I A

Patents Act 1990

PROVISIONAL SPECIFICATION

for the invention entitled:

"Combination therapy"

The invention is described in the following statement:

COMBINATION THERAPY

FIELD OF THE INVENTION

The present invention relates generally to the treatment of diseases or conditions resulting from cellular activation, such as inflammatory or cancerous diseases or conditions. In particular, the invention relates to the use of inhibitors of the cytokine or biological activity of macrophage inhibitory factor (MIF) in combination with glucocorticoids, in diseases or conditions for which treatment with a glucocorticoid is indicated.

BACKGROUND OF THE INVENTION

Glucocorticoids have been used to treat human diseases for over fifty years and are effective in a range of diseases which include inflammation, injury, ischaemia or malignancy. Although debate continues in relation to their impact on disease progression, their influence on symptoms and signs of inflammation, especially in the short term, can be dramatic.

Despite their benefits and efficacy, the use of glucocorticoids is limited by universal, predictable, dose-dependent toxicity. Mimicking Cushing's disease, a disease wherein the adrenal glands produce excess endogenous glucocorticoids, glucocorticoid treatment is associated with side effects including immunosuppression (resulting in increased susceptibility to infections), weight gain, change in body habitus, hypertension, oedema, diabetes mellitus, cataracts, osteoporosis, poor wound healing, thinning of the skin, vascular fragility, hirsutism and other features of masculinization (in females). In children, growth retardation is also noted. These side effects are known as Cushingoid side effects.

Since the side effects of glucocorticoids are dose dependent, attempts to reduce the dosage requirement have been investigated, including combination therapies in which glucocorticoids are administered with other therapeutic agents. These combination therapies are sometimes referred to as "steroid-sparing" therapies. However, currently

available combination therapies are non-specific as the other therapeutic agents do not address biological events which inhibit the effectiveness of glucocorticoids. Such combination therapies are also typically associated with serious side effects.

Furthermore, glucocorticoids are incompletely effective in a number of disease settings, leading to the concept of "steroid-resistant" diseases. Agents which amplify or enhance the effects of glucocorticoids would not only allow the reduction of dose of these agents but may also potentially render "steroid-resistant" diseases steroid-sensitive.

There is a need for effective therapies which enable a reduction in the dosage level of glucocorticoids. There is also a need for effective treatment of "steroid-resistant" diseases. Preferably, such therapies or treatments would address factors which directly limit the effectiveness of glucocorticoids.

MIF is the first identified T cell derived soluble lymphokine. MIF was first described as a soluble factor with the ability to modify the migration of macrophages[1]. The molecule responsible for the biological actions ascribed to MIF was identified and cloned in 1989[2]. Initially found to activate macrophages at inflammatory sites, it has been shown to possess pluripotential actions in the immune system. MIF has been shown to be expressed in human diseases which include inflammation, injury, ischaemia or malignancy.

MIF also has a unique relationship with glucocorticoids by overriding their anti-inflammatory effects. Unlike other pro-inflammatory cytokines, MIF is biphasically regulated by glucocorticoids, with suppression at high concentrations of glucocorticoids but induction at lower concentrations. Moreover, MIF is able to directly antagonise the effects of glucocorticoid *in vitro* on the activation of macrophages and T cells. In support of this role of MIF, MIF exerts a powerful glucocorticoid-antagonist effect *in vivo* in models including endotoxic shock and experimental arthritis. *In vitro* studies suggest that MIF acts in opposition to glucocorticoids both to affect the homeostatic state and the outcome of the inflammatory and immune response. This interaction has led to the

concept of a physiologically active "MIF-glucocorticoid dyad" that acts to regulate both normal and pathophysiological immune responses.

SUMMARY OF INVENTION

It has now been found that a combination of a compound which inhibits the cytokine or biological activity of MIF together with a glucocorticoid provides an effective therapy for diseases or conditions for which a glucocorticoid is indicated. Such a therapy may also be particularly useful in the treatment of diseases that are associated with glucocorticoid resistance.

In a first aspect, the present invention provides a method of prophylaxis or treatment of a disease or condition for which treatment with a glucocorticoid is indicated, said method comprising:

administering to a mammal a glucocorticoid and a compound which inhibits the cytokine or biological activity of macrophage inhibitory factor (MIF).

In a second aspect, the present invention provides a method of treating steroid-resistant diseases comprising:

administering to a mammal a glucocorticoid and a compound which inhibits the cytokine or biological activity of MIF.

In a third aspect, the present invention provides a method of enhancing the effect of a glucocorticoid in mammals comprising administering a compound which inhibits the cytokine or biological activity of MIF simultaneously, separately or sequentially with said glucocorticoid.

In a fourth aspect, the present invention provides a composition comprising a glucocorticoid and a compound which inhibits the cytokine or biological activity of MIF.

In a further aspect of the invention there is provided a use of a glucocorticoid in the manufacture of a medicament for administration with a compound which inhibits the cytokine or biological activity of MIF for the treatment or prophylaxis of a disease or condition for which treatment with a glucocorticoid is indicated.

In yet a further aspect of the invention there is provided a use of a compound which inhibits the cytokine or biological activity of MIF in the manufacture of a medicament for administration with a glucocorticoid for the treatment or prophylaxis of a disease or condition for which treatment of a glucocorticoid is indicated.

In yet a further aspect of the invention there is provided a use of a glucocorticoid and a compound which inhibits the cytokine or biological activity of MIF in the manufacture of a medicament for the treatment or prophylaxis of a disease or condition for which treatment with a glucocorticoid is indicated.

Preferably the amount of glucocorticoid used in the methods, uses and compositions of the invention is less than the amount which would be effective in the absence of the compound which inhibits the cytokine or biological activity of MIF. In the treatment of steroid-resistant diseases or conditions which are not responsive to glucocorticoids, any amount of glucocorticoid which is effective in combination with a compound which inhibits the cytokine or biological activity of MIF is considered less than the amount which would be effective in the absence of a compound which inhibits the cytokine or biological activity of MIF. Accordingly, the invention provides a steroid-sparing therapy.

In preferred embodiments of the invention, the glucocorticoid and compound which inhibit the cytokine or biological activity of MIF are used to treat or prevent a disease or condition in a mammal, preferably in a human subject.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will

be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

The reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that that prior art forms part of the common general knowledge in Australia.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 graphically depicts the effect of a combination of dexamethasone and benzimidazol-2-one-5-pentanoate on IL-1 induced COX-2 expression.

Figure 2 graphically depicts the effect of a combination of dexamethasone and 6,7-dihydroxynaphthalene-3-sulfonic acid on IL-1 induced COX-2 expression.

Figure 3 graphically depicts the effect of MIF antagonist, benzimidazol-2-one-5-pentanoate (MIF-a) and dexamethasone on IL-1 induced phosphorylation (activation) of ERK (extracellular kinase), as detected by Western blotting.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The mechanism through which MIF antagonises the effects of glucocorticoids has not been fully elucidated. Glucocorticoid effects on inflammation are believed to be dependent upon inhibition of specific intracellular signal-transduction pathway such as the nuclear factor κB (NF – κB) and membrane activated protein kinase (MAPK) pathways.

Without wishing to be bound by theory, it is possible that suppression of activation of these pathways by a MIF inhibitor may allow the glucocorticoid to be more effective. The MAPK pathway known as ERK (extracellular signal regulated kinase or p44/42 MAP kinase) is known to be activated by MIF[3] but has previously been regarded as resistant to the suppressive effects of glucocorticoids. It now appears that ERK activation by

interleukin-1 (IL-1) *in vitro* is resistant to suppression by the glucocorticoid dexamethasone, however, when dexamethasone is combined with a compound that inhibits the cytokine or biological activity of MIF, ERK activation is suppressed.

Notwithstanding the incomplete understanding of the pathways involved, it is possible that therapeutic antagonism of MIF removes a factor, such as ERK activation, that inhibits the anti-inflammatory effect of glucocorticoids, thereby allowing the glucocorticoids to act more effectively. It is also possible that the MIF inhibitor neutralises a natural glucocorticoid counter-regulator, allowing glucocorticoid dosages to be reduced when administered with a MIF inhibitor.

The term "disease or condition for which treatment with a glucocorticoid is indicated" refers to diseases or conditions which are capable of being treated by administration of a glucocorticoid including but not limited to autoimmune diseases, solid or haemopoitic tumours, or chronic or acute inflammatory diseases. Examples of such diseases or conditions include:

Rheumatic diseases (including but not limited to rheumatoid arthritis, osteoarthritis, psoriatic arthritis) spondyloarthropathies (including but not limited to ankylosing spondylitis, reactive arthritis, Reiter's syndrome), crystal arthropathies (including but not limited to gout, pseudogout, calcium pyrophosphate deposition disease), Lyme disease, connective tissue diseases (including but not limited to systemic lupus erythematosus, systemic sclerosis, polymyositis, dermatomyositis, Sjögren's syndrome), vasculitides (including but not limited to polyarteritis nodosa, Wegener's granulomatosis, Churg-Strauss syndrome), glomerulonephritis, inflammatory bowel disease (including ulcerative colitis, Crohn's disease), peptic ulceration, gastritis, oesophagitis, liver disease (including but not limited to cirrhosis, hepatitis), autoimmune diseases (including but not limited to diabetes mellitus, thyroiditis, myasthenia gravis, sclerosing cholangitis, primary biliary cirrhosis), pulmonary diseases (including but not limited to diffuse interstitial lung diseases, pneumoconioses, fibrosing alveolitis, asthma, bronchitis, bronchiostatis,

chronic obstructive pulmonary disease, adult respiratory distress syndrome), cancers whether primary or metastatic (including but not limited to colon cancer, lymphoma, lung cancer, melanoma, prostate cancer, breast cancer, stomach cancer, leukemia, cervical cancer and metastatic cancer), atherosclerosis (eg ischaemic heart disease, myocardial infarction, stroke, peripheral vascular disease), disorders of the hypothalamic-pituitary-adrenal axis, brain disorders (eg Alzheimers, multiple sclerosis), corneal disease, iritis, iridocyclitis, cataracts, uveitis, sarcoidosis, diseases characterised by modified angiogenesis (eg diabetic retinopathy, rheumatoid arthritis, cancer), endometrial function (menstruation, implantation, endometriosis), psoriasis, endotoxic (septic) shock, exotoxic (septic) shock, infective (true septic) shock, other complications of infection, pelvic inflammatory disease, transplant rejection, allergies, allergic rhinitis, bone diseases (eg osteoporosis, Paget's disease), atopic dermatitis, UV(B)-induced dermal cell activation (eg sunburn, skin cancer), malarial complications, diabetes mellitus, pain, inflammatory consequences of trauma or ischaemia, testicular dysfunctions and wound healing.

These diseases or conditions may also include steroid-resistant diseases or conditions where treatment with a glucocorticoid is indicated, but where the glucocorticoid is ineffective or is not as effective as expected.

The methods of the invention are preferably performed in a steroid-sparing manner. The term "steroid-sparing" refers to a combination therapy method that allows a reduction in the amount of glucocorticoid administered while still providing an effective therapy for the disease or condition being treated or prevented.

Steroid-resistant diseases or conditions are diseases or conditions for which treatment with a glucocorticoid is indicated, but where the glucocorticoid is ineffective or is not as effective as expected. This term encompasses diseases or conditions for which the effective dose of glucocorticoid results in unacceptable side effects and/or toxicity. Some steroid-resistant diseases or conditions may require a dosage of glucocorticoid so large that

they are considered non-responsive and therefore are not able to be successfully treated with glucocorticoids. Some steroid-resistant diseases or conditions may require a large dosage of glucocorticoid to achieve only a small effect on the symptoms of the disease or condition. Furthermore, some patients, diseases or conditions present with symptoms that do not respond to treatment with a glucocorticoid, or may become less sensitive to glucocorticoid treatment over time.

Glucocorticoids are a group of steroid hormones, which are used to treat or prevent a wide range of diseases or conditions. Suitable glucocorticoids may be synthetic or naturally occurring and include but are not limited to prednisolone, prednisone, cortisone acetate, beclamethasone, fluticasone, hydrocortisone, dexamethasone, methyl prednisolone, triamcinolone, budesonide and betamethasone.

A compound which inhibits the cytokine or biological activity of MIF may be a compound, protein, peptide or agent which inhibits, reduces, retards, downregulates, modifies, prevents or otherwise diminishes, wholly or partially, the functional activity of MIF.

Suitable compounds which inhibit the cytokine or biological activity of MIF include monoclonal antibodies, cinnamic acid derivatives, especially fluoro-cinnamic acid derivatives, acetaminophen derivatives[4], for example, N-acetyl-p-benzoquinone imine and hydroxy quinone derivatives, coumarins and 4-chromanones[5], aromatic amino acid schiff bases[6], naphthalenes, indanes and heterocyclic compounds such as benzimidazole derivatives, indole derivatives, benzofuran derivatives and benzothiophene derivatives, pharmaceutically acceptable salts or prodrugs thereof.

In preferred embodiments of the invention, the glucocorticoid used is selected from prednisone, prednisolone, hydrocortisone, fluticasone, beclamethasone, betamethasone, methyl prednisolone, budesonide, triamcinolone, dexamethasone and cortisone. Most preferably, the glucocorticoid is selected from prednisone, prednisolone, methyl prednisolone, fluticasone and beclamethasone. Beclamethasone and fluticasone are particularly preferred for treating asthma. Prednisone, prednisolone and methyl

prednisolone are particularly preferred in the treatment of systemic or local inflammatory diseases.

In a preferred embodiment of the invention, the compound which inhibits the cytokine or biological activity of MIF is selected from monoclonal antibody antagonists of MIF, cinnamic acids, quinone derivatives, coumarins or 4-chromanones, aromatic amino acid schiff bases, naphthalene derivatives, indane derivatives or benzimidazole, indole, benzofuran or benzothiophene derivatives. In particular, N-acetyl-p-benzoquinone imine, N-acetyl-m-hydroxy-p-benzoquinone imine, 3′,4′-dioxo-cinnamic acid, 3-substituted-7-hydroxy-coumarin and 4-chromanone derivatives, 2,6-disubstituted naphthalene compounds or 2-oxo, thio or imino substituted indanes, benzimidazoles, indoles, benzofurans and benzothiophenes and pharmaceutically acceptable salts and prodrugs thereof.

Most preferably, the compound which inhibits the cytokine or biological activity of MIF is selected from N-acetyl-p-benzoquinone imine, N-acetyl-m-hydroxy-p-benzoquinone imine, 3′,4′-dioxo-cinnamic acid, 3-substituted-7-hydroxy-coumarins and 4-chromanone derivatives, 2,6-disubstituted naphthalene compounds or 2-oxo, thio or imino substituted benzimidazoles and pharmaceutically acceptable salts and prodrugs thereof. In particular, N-acetyl-p-benzoquinone imine, N-acetyl-m-hydroxy-p-benzoquinone imine, 3′,4′-dioxo-cinnamic acid, 6,7-dimethoxy-2-naphthoic acid, 7-hydroxynaphthalene-3-sulfonic acid, 6,7-dihydroxynaphthalene-3-sulfonic acid, 3-carboxy-7-hydroxynaphthalene-8-sulfonic acid, 5-methylbenzimidazol-2-one, benzimidazol-2-one-5-carboxylic acid, benzimidazol-2-one-5-n-pentanoate, 5-[2-(1-oxy-2-hydroxyethyl)ethyl]benzimidazol-2-one-5-carboxylate, benzimidazol-2-one-5-methanoate, benzimidazol-2-one-5-ethanoate and benzimidazol-2-one-5-carboxylate-amino acid or dopamine adducts, and their derivatives and pharmaceutically acceptable salts and prodrugs thereof.

As used herein, MIF includes human or other animal MIF and derivatives and naturally occurring variants thereof which at least partially retain MIF cytokine or biological activity. Thus, the subject to be treated may be human or other animal such as a mammal.

Non-human subjects include, but are not limited to primates, livestock animals (eg sheep, cows, horses, pigs, goats), domestic animals (eg dogs, cats), birds and laboratory test animals (eg mice rats, guinea pigs, rabbits).

Reference herein to "cytokine or biological activity" of MIF includes the cytokine or biological effect on cellular function via autocrine, endocrine, paracrine, cytokine, hormone or growth factor activity, or via intracellular effects.

The amounts of glucocorticoid and compound which inhibits the cytokine or biological activity of MIF are selected such that in combination they provide complete or partial treatment or prophylaxis of a disease or condition for which a glucocorticoid is indicated. The amount of compound which inhibits the cytokine or biological activity of MIF is preferably an amount that will at least partially inhibit the cytokine or biological activity of MIF. The amount of glucocorticoid is preferably less than the amount required in the absence of the compound which inhibits the cytokine or biological activity of MIF. The amounts of glucocorticoid and compound which inhibits the cytokine or biological activity of MIF used in a treatment or therapy are selected such that in combination they at least partially attain the desired therapeutic effect, or delay onset of, or inhibit the progression of, or halt or partially or fully reverse the onset or progression of the disease or condition being treated. The amounts of glucocorticoid and compound which inhibits the cytokine or biological activity of MIF used in the prophylaxis of a disease or condition are selected such that in combination they at least partially prevent or delay the onset of the disease or condition. Dosing may occur at intervals of minutes, hours, days, weeks, months or years or continuously over any one of these periods.

Suitable dosages of a compound which inhibits the cytokine or biological activity of MIF may lie within the range of about 0.1 ng per kg of body weight to 1 g per kg of body weight per dosage. The dosage is preferably in the range of 1 μg to 1 g per kg of body weight per dosage, such as is in the range of 1 mg to 1 g per kg of body weight per dosage. In one embodiment, the dosage is in the range of 1 mg to 500 mg per kg of body weight per dosage. In another embodiment, the dosage is in the range of 1 mg to 250 mg per kg of

body weight per dosage. In yet another preferred embodiment, the dosage is in the range of 1 mg to 100 mg per kg of body weight per dosage, such as up to 50 mg per kg of body weight per dosage. In yet another embodiment, the dosage is in the range of 1μg to 1mg per kg of body weight per dosage.

Suitable dosage amounts of glucocorticoids will depend, in part, on the mode of administration and whether the dosage is being administered in a single, daily or divided dose, or as a continuous infusion. When administered orally, intravenously, intramuscularly, intralesionally or intracavity (eg. intra-articular, intrathecal, intrathoracic), dosages are typically between 1 mg to 1000 mg, preferably 1 mg to 100 mg, more preferably 1 mg to 50 mg or 1 mg to 10 mg per dose. When administered topically or by inhalation as a single, daily or divided dose, dosages are typically 1 ng to 1 μg, 1 ng to 1 mg or 1 pg to 1 μg.

Suitable dosage amounts and dosing regimens can be determined by the attending physician or veterinarian and may depend on the desired level of inhibiting activity, the particular condition being treated, the severity of the condition as well as the general age, health and weight of the subject.

The glucocorticoid and compound which inhibits the cytokine or biological activity of MIF may be administered simultaneously or sequentially. The active ingredients may be administered alone but are preferably administered as a pharmaceutically acceptable composition or separate pharmaceutically acceptable compositions.

The formulation of such compositions is well known to those skilled in the art. The composition or compositions may contain pharmaceutically acceptable additives such as carriers, diluents or excipients. These include, where appropriate, all conventional solvents, dispersion agents, fillers, solid carriers, coating agents, antifungal and antibacterial agents, dermal penetration agents, surfactants, isotonic and absorption agents and the like. It will be understood that the compositions of the invention may also include other supplementary physiologically active agents.

The carrier must be pharmaceutically acceptable in the sense of being compatible with the other ingredients of the composition and not injurious to the subject. Compositions include those suitable for oral, rectal, inhalational, nasal, transdermal, topical (including buccal and sublingual), vaginal or parenteral (including subcutaneous, intramuscular, intraspinal, intravenous and intradermal) administration. The compositions may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. Such methods include the step of bringing into association the active ingredient or ingredients with the carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and then if necessary shaping the product.

Depending on the disease or condition to be treated, it may or may not be desirable for glucocorticoids and/or compounds which inhibit the cytokine or biological activity of MIF to cross the blood/brain barrier. Thus the compositions for use in the present invention may be formulated to be water or lipid soluble.

Compositions of the present invention suitable for oral administration may be presented as discrete units such as capsules, sachets or tablets each containing a predetermined amount of the active ingredient; as a powder or granules; as a solution or a suspension in an aqueous or non-aqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion. The active ingredients may also be presented as a bolus, electuary or paste.

A tablet may be made by compression or moulding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredient in a free-flowing form such as a powder or granules, optionally mixed with a binder (eg inert diluent, preservative, disintegrant (eg. sodium starch glycolate, cross-linked polyvinyl pyrrolidone, cross-linked sodium carboxymethyl cellulose)) surface-active or dispersing agent. Moulded tablets may be made by moulding in a

suitable machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets may optionally be coated or scored and may be formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropylmethyl cellulose in varying proportions to provide the desired release profile. Tablets may optionally be provided with an enteric coating, to provide release in parts of the gut other than the stomach.

Compositions suitable for topical administration in the mouth include lozenges comprising the active ingredient in a flavoured base, usually sucrose and acacia or tragacanth gum; pastilles comprising the active ingredient in an inert basis such as gelatin and glycerin, or sucrose and acacia gum; and mouthwashes comprising the active ingredients in a suitable liquid carrier.

The glucocorticoids and/or compounds which inhibit the cytokine or biological activity of MIF may also be administered intranasally or via inhalation, for example by atomiser, aerosol or nebulizer means.

Compositions suitable for topical administration to the skin may comprise the glucocorticoids and/or compound which inhibit the cytokine or biological activity of MIF dissolved or suspended in any suitable carrier or base and may be in the form of lotions, gel, creams, pastes, ointments and the like. Suitable carriers include mineral oil, propylene glycol, polyoxyethylene, polyoxypropylene, emulsifying wax, sorbitan monostearate, polysorbate 60, cetyl esters wax, cetearyl alcohol, 2-octyldodecanol, benzyl alcohol and water. Transdermal devices, such as patches, may also be used to administer the glucocorticoids and/or compound which inhibit the cytokine or biological activity of MIF.

Compositions for rectal administration may be presented as a suppository with a suitable carrier base comprising, for example, cocoa butter, gelatin, glycerin or polyethylene glycol.

Compositions suitable for vaginal administration may be presented as pessaries, tampons, creams, gels, pastes, foams or spray formulations containing, in addition to the glucocorticoids and/or compound which inhibit the cytokine or biological activity of MIF, such carriers as are known in the art to be appropriate.

Compositions suitable for parenteral administration include aqueous and non-aqueous isotonic sterile injection solutions which may contain anti-oxidants, buffers, bactericides and solutes which render the composition isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The compositions may be presented in unit-dose or multi-dose sealed containers, for example, ampoules and vials, and may be stored in a freeze-dried (lyophilised) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

Preferred unit dosage compositions are those containing a daily dose or unit, daily sub-dose, as herein above described, or an appropriate fraction thereof, of the glucocorticoids and/or compound which inhibit the cytokine or biological activity of MIF.

It should be understood that in addition to the active ingredients particularly mentioned above, the compositions of this invention may include other agents conventional in the art having regard to the type of composition in question, for example, those suitable for oral administration may include such further agents as binders, sweeteners, thickeners, flavouring agents, disintegrating agents, coating agents, preservatives, lubricants and/or time delay agents. Suitable sweeteners include sucrose, lactose, glucose, aspartame or saccharine. Suitable disintegrating agents include corn starch, methylcellulose, polyvinylpyrrolidone, xanthan gum, bentonite, alginic acid or agar. Suitable flavouring agents include peppermint oil, oil of wintergreen, cherry, orange or raspberry flavouring. Suitable coating agents include polymers or copolymers of acrylic acid and/or methacrylic acid and/or their esters, waxes, fatty alcohols, zein, shellac or gluten. Suitable

preservatives include sodium benzoate, vitamin E, alpha-tocopherol, ascorbic acid, methyl paraben, propyl paraben or sodium bisulphite. Suitable lubricants include magnesium stearate, stearic acid, sodium oleate, sodium chloride or talc. Suitable time delay agents include glyceryl monostearate or glyceryl distearate.

The methods and compositions of the present invention may also be presented for use in veterinary applications. Single or separate compositions may be prepared by any suitable means known in the art. Examples of such compositions, including single compositions containing both a glucocorticoid and a compound which inhibits the cytokine or biological activity of MIF, and compositions containing each active agent separately, include those adapted for

(a) oral administration, external application (eg drenches including aqueous and non-aqueous solutions or suspensions), tablets, boluses, powders, granules, pellets for admixture with feedstuffs, pastes for application to the tongue;

(b) parenteral administration, eg subcutaneous, intramuscular or intravenous injection as a sterile solution or suspension; and

(c) topical application eg creams, ointments, gels, lotions, etc.

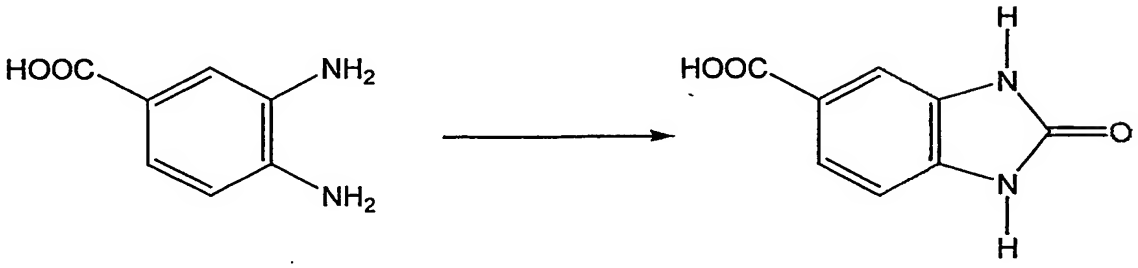
Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications which fall within the spirit and scope. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

The invention will now be described with reference to the following examples which are included for the purpose of illustration only and are not intended to limit the generality of the invention hereinbefore described.

EXAMPLES

EXAMPLE 1:

Example 1A: ***Preparation of Benzimidazol-2-one-5-carboxylic acid***



The method described by *Harvey et al* [7] for the preparation of 5-methylbenzimidazol-2-one was used to prepare benzimidazol-2-one-5-carboxylic acid except this preparation started with 3,4-diaminobenzoic acid.

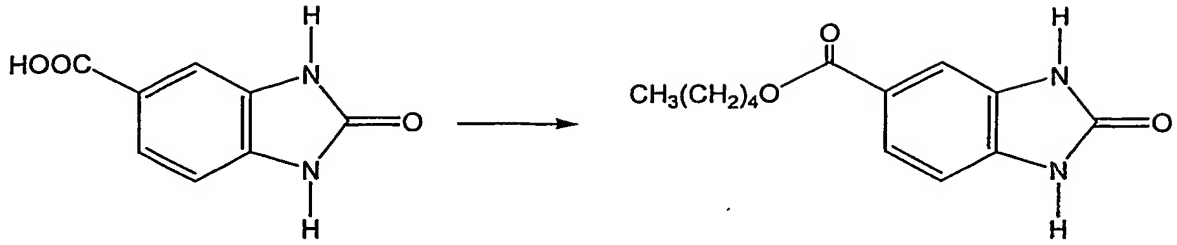
Urea (1.20 g, 0.0200 mol) and 3,4-diaminobenzoic acid (3.04 g, 0.0200 mol) in pentan-1-ol (10 mL) was vigorously stirred and heated to reflux under a nitrogen atmosphere. The heating was discontinued after 4 hours and on cooling to room temperature, water (30 mL) was added. The pH was adjusted to 1 with conc. HCl. The resultant dark solid was filtered off, washed with further water (2 x 20 mL) and dried to give 3.00 g (84% yield) of benzimidazol-2-one-5-carboxylic acid as a black powder;

R_f: 0.09 (9:1 $CHCl_3$:MeOH), 0.20 (4:1 $CHCl_3$:MeOH),

^{1}H NMR (d_6-DMSO): δ 6.98 (d, 1 H, $J_{7,6}$ 8.1 Hz, H-7), 7.45(d, 1 H, $J_{4,6}$ 1.2 Hz, H-4), 7.60 (dd, 1 H, H-6), 10.78 (bs, 1 H, NH), 10.94 (bs, 1 H, NH);

LRESI negative ion mass spectrum: *m/z* 177 (100%, M-H$^-$).

Benzimidazol-2-one-5-n-pentanoate



Benzimidazol-2-one-5-carboxylic acid (250 mg, 0.9070 mmol) prepared as described above and Dowex 50W-X8(H^+) resin (250 mg) were suspended in pentan-1-ol (40 mL) and the mixture heated to reflux for 42 hours. The solid was filtered off and washed with methanol (3 x 20 mL) and the combined filtrates evaporated to dryness to give benzimidazol-2-one-5-*n*-pentanoate (310 mg, 43% yield) as an off-white powder;

R_f: 0.63 (4:1 $CHCl_3$:MeOH),

mp: 227-228°C,

^1H NMR ($CDCl_3/CD_3OD$): δ 0.88-0.92 (pseudo t, 3 H, CH_3), 1.33-1.43 (m, 4 H, 2 x CH_2), 1.70-1.79 (m, 2 H, CH_2), 4.25-4.29 (pseudo t, 2 H, CH_2), 7.04 (d, 1 H, $J_{7,6}$ 8.4 Hz, H-7), 7.44 (bs, 1 H, NH), 7.55 (bs, 1 H, NH), 7.66 (bs, 1 H, H-4), 7.75 (dd, 1 H, $J_{6,5}$ 1.5 Hz, H-6);

LRESI negative ion mass spectrum: *m/z* 247 (100%, $[M-H]^-$);

HRESI positive ion mass spectrum: $C_{13}H_{17}N_2O_3$ calculated 249.12391,

$C_{13}H_{16}N_2O_3$ calculated C, 62.97; H, 6.50; N, 11.29, found C, 63.1, H, 6.54, N, 11.05.

Example 1B: ***In vitro assay of MIF antagonism by benzimidazol-2-one-5-carboxylic acid***

The activity of benzimidazol-2-one-5-carboxylic was studied in a bioassay utilising MIF-dependent activation of human dermal fibroblasts. Sampey *et al*[8] have shown that induction of the expression of cyclooxygenase-2 (COX-2) by the cytokine interleukin 1 (IL-1) is dependent upon the presence of MIF, i.e. can be prevented using specific anti-MIF monoclonal antibody. IL-1-induced COX-2 expression is therefore a MIF-dependent event.

S112 human dermal fibroblasts were propagated in RPMI/10% foetal calf serum (FCS). Prior to experimentation, cells were seeded at 10^5 cells/ml in RPMI/0.1% BSA for 18 hours. Cells were treated with recombinant human IL-1 (0.1 ng/ml) and with benzimidazol-2-one-5-carboxylic acid at 1-100 μM. A control was treated only with recombinant human IL-1 (0.1 ng/ml). After 6 hours, cells were collected and intracellular COX-2 protein determined by permeabilisation flow cytometry. Cells permeabilised with 0.1% saponin were sequentially labelled with a mouse anti-human COX-2 monoclonal antibody and with sheep-anti-mouse F(ab)2 fragment labelled with fluoroscein isothiocyanate. Cellular fluorescence was determined using a flow cytometer. At least 5000 events were counted for each reading, each of which was performed in duplicate, and the results expressed in mean fluorescence intensity (MFI) after subtraction of negative control-labelled cell fluorescence.

The effect of benzimidazol-2-one-5-carboxylic acid was determined by subtracting the IL-1+benzimidazol-2-one-5-carboxylic acid-treated cell MFI from the IL-1-treated cell (control) MFI and expressed as % inhibition.

The average % inhibition over 9 experiments for benzimidazol-2-one-5-carboxylic acid (50 μM) was –43.4%.

The dose response curves for benzimidazol-2-one-5-carboxylic acid observed in 3 experiments where benzimidazol-2-one-5-carboxylic acid was added in 1 μM, 10 μM, 50 μM and 100 μM quantities and the samples analysed for IL-1 induced COX-2 expression as described above is shown in Table 1.

Table 1

Concentration (μM)	Average % inhibition
1	0
10	16.9
50	51.1
100	63.1

Example 1C: ***In vivo assay of MIF antagonism of benzimidazol-2-one-5-n-pentanoate***

The activity of benzimidazol-2-one-5-n-pentanoate was studied in the murine endotoxic shock model. This model has been previously shown to be dependent on MIF[9]. Endotoxaemia was induced by intra-peritoneal injection of lipopolysaccharide (LPS) (15mg/kg) in 400 µl saline. Mice were treated with a saline solution (control) only, a saline solution and LPS or benzimidazol-2-one-5-n-pentanoate at a dose of 15 mg/kg body weight by intra-peritoneal injection at 24 hours, 12 hours and 1 hour before intra-peritoneal LPS injection. After 24 hours mice were humanely killed by CO_2 inhalation then neck dislocation. Serum was obtained from blood obtained by cardiac puncture prior to death and measured for cytokines including interleukin 1 (IL-1) and interleukin 6 (IL-6) by ELISA. The production of IL-1 and IL-6 has been previously shown to be dependent on MIF[10]. Macrophages were obtained by lavage of the peritoneal cavity using normal saline and placed into 24 well tissue culture plates for 18 hours in RPMI/10%FCS. The cultured peritoneal macrophage supernatants were then analysed for cytokines including IL-6. The peritoneal lavage supernatants were also analysed for cytokines including IL-6. The results are provided in Table 2.

Table 2

Experiment	Serum IL-6 (ng/ml)	Serum IL-1 (ng/ml)	Cultured peritoneal macrophage IL-6 (ng/ml)	Peritoneal lavage IL-6 (ng/ml)
control	8.81	0	3.39	0
LPS only	26.05	582	3.40	16.11
LPS + benzimidazol-2-one-5-n-pentanoate	99.78	257	1.33	7.07

Example 1D: ***In vitro toxicity assay for benzimidazol-2-one-5-n-pentanoate***

Benzimidazol-2-one-5-n-pentanoate has low toxicity towards cells. The toxicity of benzimidazol-2-one-5-n-pentanoate was examined *in vitro* to assess cytotoxicity. Human

dermal fibroblast cell line (S112) cells were exposed to vehicle (control), benzimidazol-2-one-5-n-pentanoate (50 μM) or sodium nitroprusside (SNP) (0.5 μM). SNP is a positive control agent which induces dose-dependent apoptosis in S112 cells. Toxicity was assessed by analysis of apoptosis using flow cytometric detection of cell surface Annexin V binding and propidium iodide staining. At least 5000 events were analysed for each experiment. Cells positive for both Annexin V and propidium iodide were designated as apoptotic and cells negative for both Annexin V and propidium iodide were designated as viable. Results are expressed as the percentage (%) of cells with each of these labels. Benzimidazol-2-one-5-n-pentanoate did not induce apoptosis at levels above the control whereas SNP induced a high level of apoptosis. The results for benzimidazol-2-one-5-n-pentanoate are shown in Table 3.

Table 3

	Control	benzimidazol-2-one-5-n-pentanoate (50μm)	SNP(0.5μm)
viable cells	80.2	80.1	20.7
apoptotic cells	15.1	16.8	65.1

Example 1E: ***In vitro assay of MIF antagonism in presence of glucocorticoid.***

The above *in vitro* assay for analysing IL-1 induced COX-2 expression given in Example 1B was repeated using benzimidazol-2-one-5-n-pentanoate (50 μM), dexamethasone (10^{-9} M) or a combination of dexamethasone (10^{-9} M) and benzimidazol-2-one-5-n-pentanoate (50 μM). The results are shown in Table 4 and Figure 1.

Table 4

Experiment	Compound	% Inhibition
1	benzimidazol-2-one-5-n-pentanoate	-45.1
2	Dexamethasone	-46.8
3	benzimidazol-2-one-5-n-pentanoate + dexamethasone	-73.6

EXAMPLE 2:

6,7-dihydroxynaphthalene-2-sulfonic acid was obtained from Aldrich (Catalogue No. 21,896-0)

Example 2A: ***In vitro assay of MIF antagonism***

The activity of 6,7-dihydroxynaphthalene-2-sulfonic acid was studied in a bioassay utilising MIF-dependent activation of human dermal fibroblasts as described in Example 1B above.

The % average inhibition of COX-2 over 9 experiments for 6,7-dihydroxynaphthalene-2-sulfonic acid was –25.4%.

The dose response for 6,7-dihydroxynaphthalene-3-sulphonic acid at a concentration of 0.01, 0.1, 1.0, 10 and 50 μM is given in Table 5.

Table 5

Concentration (μM)	% inhibition
0.01	-5
0.1	-10
1	-5.5
10	-19.5
100	-25.4

Example 2B: ***In vivo assay of MIF antagonism***

The activity of 6,7-dihydroxynaphthalene-3-sulfonic acid was studied in the murine endotoxic shock model as described in Example 1C above without analysis of cultured peritoneal macrophage or peritoneal lavage supernatants. Table 6 shows analysis of serum IL-1 (ng/ml) and IL-6 when LPS is administered alone or in combination with 6,7-dihydroxynaphthalene-3-sulfonic acid.

Table 6

Experiment	Serum IL-6 (ng/ml)	Serum IL-1 (ng/ml)
control	8.8	101.0
LPS only	97.1	426.5
LPS + 6,7-dihydroxynaphthalene -3-sulfonic acid	48.5	257.7

Example 2C: ***In vitro toxicity assay for 6,7-dihydroxynaphthalene-3-sulfonic acid***

6,7-Dihydroxynaphthalene-3-sulfonic acid has low toxicity towards cells. The toxicity of the this compound was examined *in vitro* to assess cytotoxicity as described in Example 1D above. Results are expressed as the percentage (%) of cells with each of these labels. 6,7-Dihydroxynaphthalene-3-sulfonic acid did not induce apoptosis at levels above the control. The results for 6,7-dihydroxynaphthalene-3-sulfonic acid are shown in Table 7.

Table 7

	Control	6,7-Dihydroxynaphthalene-3-sulfonic acid (50μm)
viable cells	80.1	83.0
apoptotic cells	14.3	14.2

Example 2D: ***In vitro assay of MIF antagonism in presence of glucocorticoid.***

The above *in vitro* assay for analysing IL-1 induced COX-2 expression given in Example 1E was repeated using 6,7-dihydroxynaphthalene-3-sulfonic acid (50 μM), dexamethasone (10^{-9} M) or a combination of dexamethasone (10^{-9} M) and 6,7-dihydroxynaphthalene-3-sulfonic acid (50 μM). The results are shown in Table 8 and Figure 2.

Table 8

Experiment	Compound	% inhibition
1	6,7-dihydroxynaphthalene sulfonic acid	-38.0
2	dexamethasone	-63.8
3	6,7-dihydroxynaphthalene sulfonic acid and dexamethasone	-83.3

Example 3:

S112 human dermal fibroblasts cultured in RPMI (serum-free) were stimulated with recombinant human IL-1 0.1 ng/ml, as described herein, for 30 minutes, with or without the addition of dexamethasone 10^{-9}M or benzimidazol-2-one-5-pentanoate (50 μM). The phosphorylation (activation) of ERK was assessed using Western blotting with a mAb specific for the phosphorylated (activated) form of ERK. In brief, cells were disrupted by repeated aspiration through a 21-gauge needle. After incubation on ice for 10 min and microcentrifugation at 3000 rpm for 15 min (4°C), the supernatants were removed, the protein concentration was determined, and the lysates were stored at –80°C. Equal amounts of cellular proteins were fractionated on 10% SDS-polyacrylamide electrophoresis gels and transferred to polyvinylidene difluoride membranes. Immunoblotting was performed using antibodies directed against phospho-p44/42 (ERK) and total p44/42 according to the manufacturer's instructions. The intensity of ERK activation is proportional to the size and optical density (darkness) of the resulting blots. Total ERK blots serve as a loading control, such that changes in phosphorylated ERK represent changes in phosphorylation and not in total ERK protein. In these experiments, ERK activation by the pro-inflammatory cytokine IL-1 was not inhibited by either benzimidazol-2-one-5-pentanoate or dexamethasone alone, but was powerfully inhibited by the combination of benzimidazol-2-one-5-pentanoate and dexamethasone. The results are shown in Figure 3.

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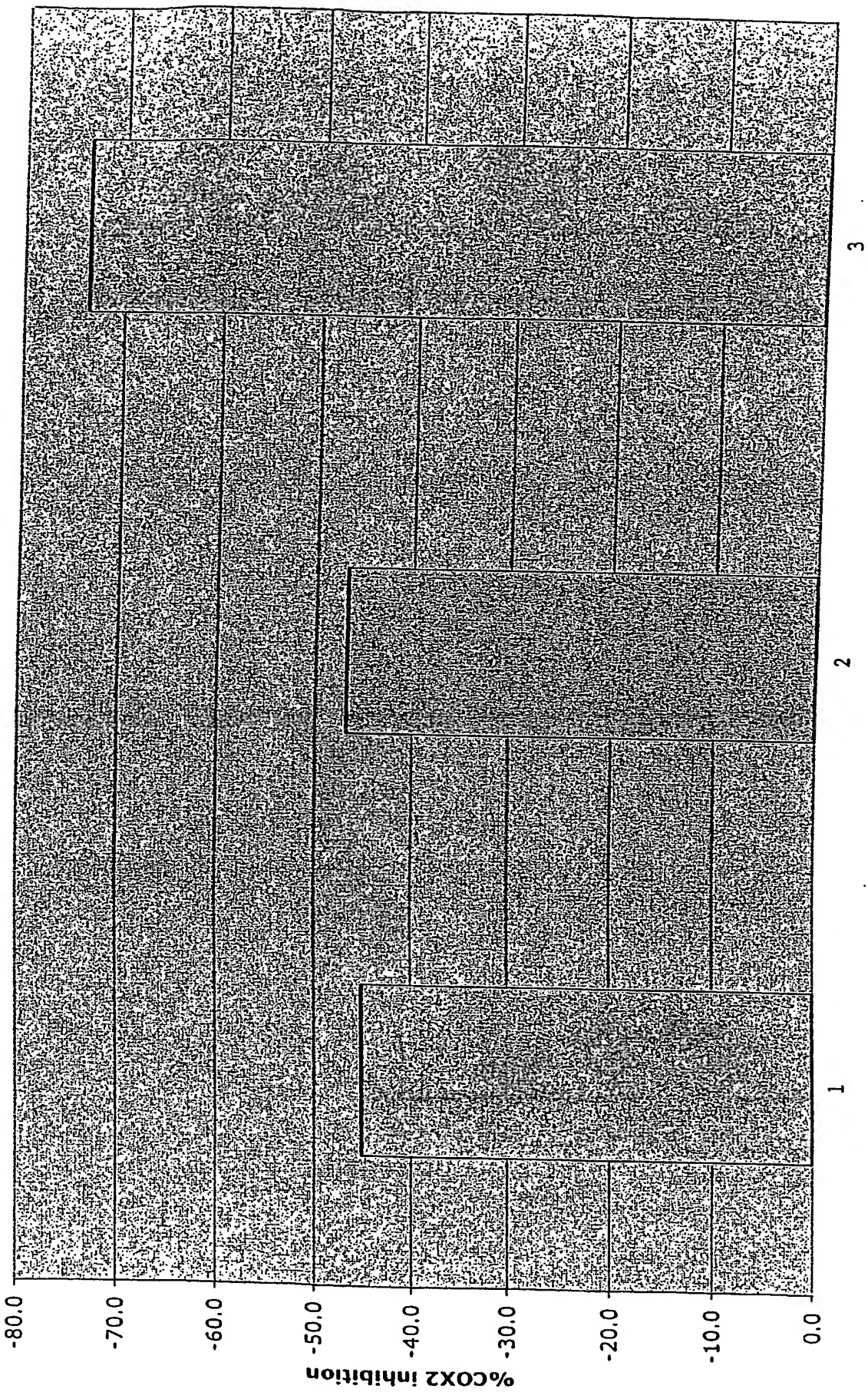
Figure 1



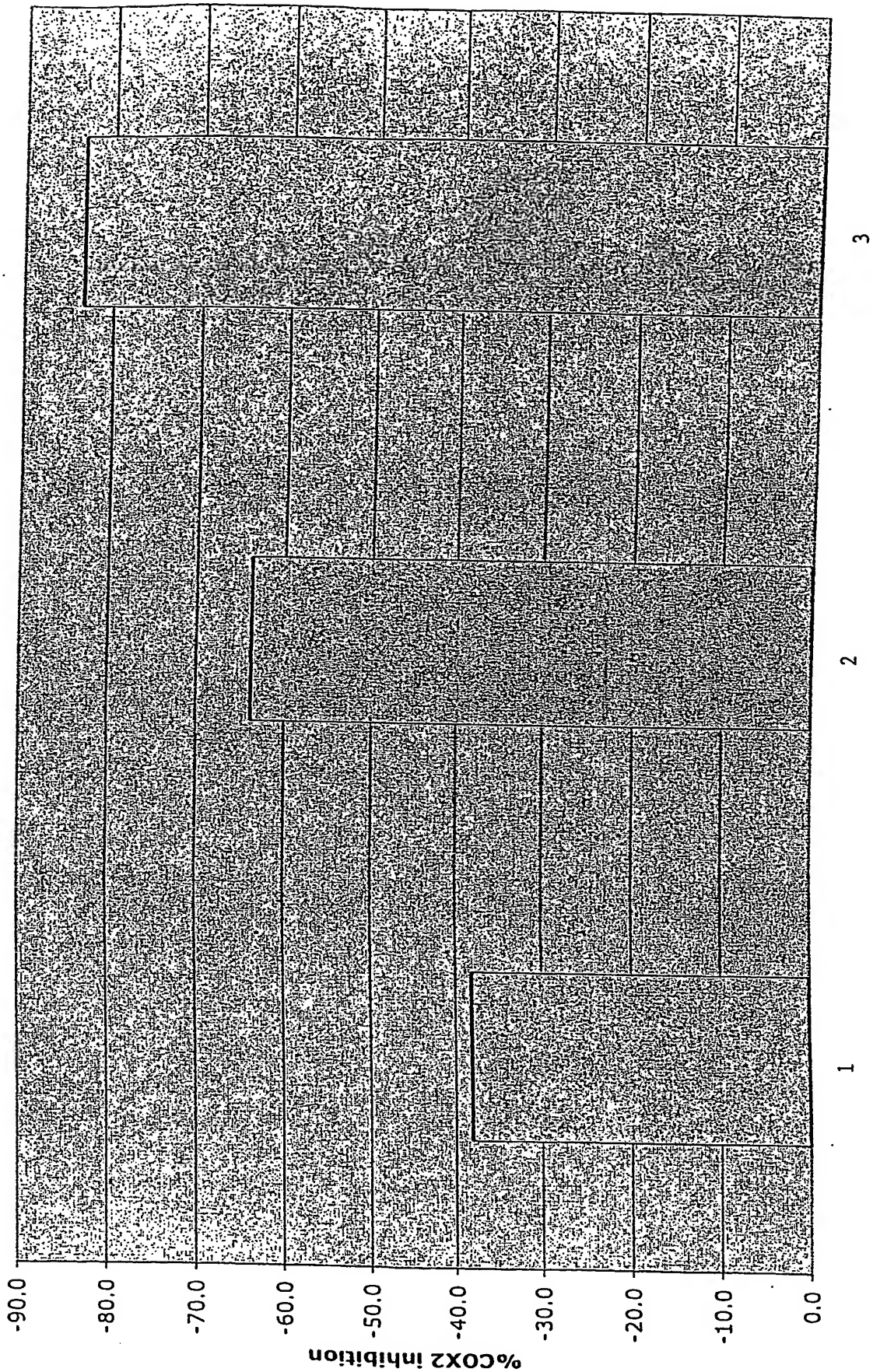
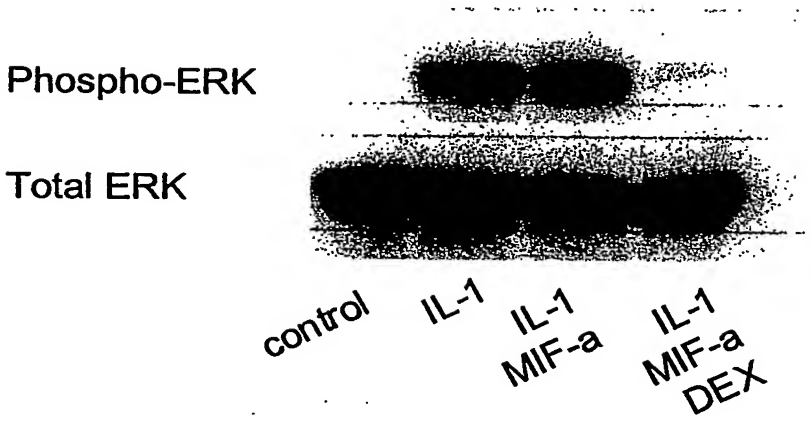


Figure 2

Figure 3



*IL-1: Interleukin 10.1 ng/ml
MIF-a: benzimidazole-2-one-5-pentanoate 50μM
DEX: dexamethasone 10^{-9}M